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Alogliptin improves endothelial function by promoting autophagy in perivascular adipose tissue of obese mice through a GLP-1-dependent mechanism

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ABSTRACT

Objective: Perivascular adipose tissue (PVAT) regulates vascular function in a paracrine manner and the vasodilatory effect of PVAT on vessels is completely abolished in obesity. In addition, autophagy is required for maintaining biological function of PVAT and has been shown to be inhibited in obesity. The aim of this study was to explore whether alogliptin improves endothelial function by promoting autophagy in PVAT in obese mice.
Methods: C57BL/6 mice were maintained on high fat diet with or without alogliptin intervention for 3 months. Vasorelaxation function of thoracic aorta with or without PVAT was determined. Autophagy related protein level of p62 and LC3B, along with phosphorylated mTOR (p-mTOR) were evaluated. In addition, the effects of alogliptin on autophagy were also investigated in cultured adipocytes.
Results: The presence of PVAT significantly impaired endothelium-dependent vasodilation in obese mice and alogliptin intervention corrected this defect. Autophagy in PVAT was decreased in obese mice and alogliptin intervention activated autophagy. Activating autophagy in PVAT improved endothelium-dependent vasodilation while blocking it in PVAT impaired vasodilation function. Further, addition of glucagon-like peptide-1 (GLP-1) but not alogliptin alone activated autophagy. Moreover, GLP-1 and alogliptin co-treatment did not show additive effect on activating autophagy.
Conclusions: These results revealed that promoting autophagy in PVAT improved endothelial function in response to alogliptin intervention. Additionally, the beneficial effect of alogliptin intervention on PVAT was GLP-1 dependent.

1. Introduction

Perivascular adipose tissue (PVAT), which surrounds most of blood vessels in the body, is an endocrine tissue that secretes several adipokines which regulate and maintain vascular tone through endothelial-dependent and endothelial-independent mechanisms [1]. Under physiological conditions, PVAT exerts anti-contractile function on the vessels. However, the vasodilatory effect of PVAT is completely lost in obesity [2]. PVAT becomes dysfunctional in obesity expresses inflammatory profiles and diminishes local adiponectin and nitric oxide (NO) bioavailability [3], further leading to endothelial dysfunction [4]. Approaches that restore the function of PVAT may contribute in improving endothelial function.

Autophagy is an evolutionarily conserved intracellular catabolic mechanism that delivers damaged organelles and macromolecules to lysosomes for degradation, which is critical for homeostasis. It is known that autophagy is activated in nutrient deprivation and suppressed in over-nutrition conditions and obesity [5]. It has been proposed that cytoprotective autophagy is necessary for the formation and breakdown of lipid droplets [6,7]. Conversely, disruption of autophagy leads to

Abbreviations: PVAT, perivascular adipose tissue; DPP-4, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; NO, nitric oxide; NE, norepinephrine; Ach, acetylcholine; SNP, sodium nitroprusside; PA, palmitic acid; RAPA, rapamycin; CQ, chloroquine; bs, bath solution; ELISA, enzyme-linked immunosorbent assay; TG, triglycerides; TC, total cholesterol; FFA, free fatty acid; TNF-α, tumor necrosis factor-α

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obesity [8] and obesity-related disorders [9]. Therefore, dysregulation of autophagy may be an underlying mechanism of PVAT disfunction.

Alogliptin, a highly-selective dipeptidyl peptidase-4 (DPP-4) inhibitor, is used to improve glycemic control in type 2 diabetic mellitus. DPP-4, a serine protease, is present in plasma in a soluble form and is also expressed in a variety of cell types [10]. The peptidase rapidly degrades incretins (mainly including GLP-1, that regulate glucose homeostasis) into inactive forms. Recent studies found DPP-4 inhibitors and GLP-1 receptor (GLP-1R) agonists exert pleiotropic effects on regulating obesity-associated disorders, such as attenuating obesity-related inflammation [11], and improving hepatic steatosis [12,13]. Of note, some researches have proved that GLP-1 related interventions could induce autophagy under high-calorie condition [12,14,15].

Collectively, these findings indicate that dysfunctional PVAT fails to protect vascular function in obesity. Therefore, it is plausible that activating autophagy in PVAT would help to regulate the function of PVAT and thereby improve vascular function in obesity. In this study, we explored whether alogliptin intervention activated autophagy in PVAT in mice on a high fat diet, and if that improved vascular function.

2. Materials and methods

2.1. Animals models

Animal procedures conformed to the National Institutes of Health Guidelines for the Use of Laboratory Animals and were approved by the Animal Ethics Committee of Wuhan General Hospital affiliated to Southern Medical University. Twelve-month-old male C57BL/6J mice were maintained on normal chow (NC; $n = 35$ mice), high fat diet (HFD, 60% of calories from fat; $n = 35$ mice) or alogliptin plus HFD (AHF, $n = 35$ mice). The dose of alogliptin was estimated from previous studies [16,17]. Alogliptin was purchased from Takeda Co. (Japan) and mixed to homogeneity during manufacturing of the diets (Huafukang Co., China). Body weight was measured on a weekly basis during period of the study. After 3 months of intervention, all mice were fasted overnight, and then anesthetized by intraperitoneally (i.p.) injection of pentobarbital sodium (60 mg/kg) and euthanized for tissues samples.

2.2. Aorta vasorelaxation function assessment

Vasorelaxation was assayed as previously described [18,19]. Briefly, thoracic aortas with or without PVAT were cut into approximate 4 mm rings and suspended in Krebs buffer (37 °C, 95% O_2 and 5% CO_2). After equilibrated with 0.5 *g* tension for 1 h, the rings were contracted with norepinephrine (NE, 10^{-6} mmol/L). When steady state was reached, vasodilation responses were determined by cumulative concentration-response curves to acetylcholine (Ach, 10^{-9} to 10^{-4} mmol/L) and sodium nitroprusside (SNP, 10^{-9} to 10^{-4} mmol/L).

2.3. Bath solution transfer experiments

PVAT from thoracic aortas was dissected under stereo-microscope. (1) For *ex vivo* experiments, we pre-treated PVAT with exendin 9-39 (100 nmol/L, a GLP-1R antagonist), rapamycin (300 nmol/L, autophagy activator) or chloroquine (20 μmol/L, autophagy inhibitor) for 10 min, followed by addition of alogliptin (50 μmol/L) for 10 min, finally GLP-1 (100 nmol/L), if indicated and PVAT was incubated for 2 h. (2) For *in vitro* experiments, we pretreated PVAT with palmitic acid (PA, 500 μmol/L) for 10 min, then added exendin 9-39 (100 nmol/L) or chloroquine (CQ, 20 μmol/L) for 10 min, and finally added GLP-1 (100 nmol/L) where indicated and incubated PVAT for 2 h. After washing with PBS, PVAT was incubated in DMEM medium (Gibco, USA) for 22 h. Then, the medium was collected as bath solution (bs). Subsequently, the bath solution was used to incubate recipient aorta without PVAT for an initial 60 min, and vasorelaxation function was assayed as described above. Adiponectin and TNF-α levels in bath solution were measured by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA).

2.4. Western blotting

Protein samples from PVAT of 15 months old mice or cell extracts were analyzed by Western blotting as described previously [20]. Antibodies used were: p62 (dilution: 1:1000, #5114, Cell Signaling Technologies), LC3B (dilution: 1:2000, Ab192890, Abcam), p-mTOR (dilution: 1:600, BS4706, Bioworld), mTOR (dilution: 1:600, BS3611, Bioworld), p-Akt (dilution: 1:2000, #4060, Cell Signaling Technologies), Akt (dilution: 1:1000, #9272, Cell Signaling Technologies), p-eNOS (dilution: 1:1000, #9571, Cell Signaling Technologies), eNOS (dilution: 1:1000, #9572, Cell Signaling Technologies), and β-actin (dilution: 1:200, BM0627, Boster Bioengineering Co., Wuhan, China). Blots were quantified with ImageJ software.

2.5. Immunohistochemistry

Thoracic aortas with PVAT were fixed in 4% paraformaldehyde for 48 h and paraffin-embedded. Consecutive 4 μm sections were de-waxed, rehydrated, and immunostained for adiponectin (dilution: 1:100, bs-0471R, Bioss) and TNF-α (dilution: 1:200, Ab6671, Abcam) as previously reported [3].

2.6. Flow cytometry

3T3-L1 preadipocytes were purchased from Cell Resource Center of Peking Union Hospital. 3T3-L1 preadipocytes were differentiated to adipocytes as previously reported [21]. Adipocytes in various groups were infected with adenovirus encoding mRFP-GFP-LC3 (10^{10} PFU/ml; Hanbio, Shanghai, China) at 30 multiplicities of infection 48 h before assay. Autophagosomes were labeled with both red and green signal (mRFP-GFP) and autolysosomes with red signal (mRFP). Autophagic flux was evaluated by mean fluorescence intensity (MFI) of mRFP/GFP using flow cytometry.

2.7. Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted in cultured adipocytes and reverse-transcribed to cDNA as previously described [22]. Relative changes in mRNA of various groups were determined with 2ΔΔCt method. Primers for qPCR were adiponectin, 5′-GGAGAGAAAGGAGATGCAGGT-3′ and 5′-CTTTCCTGCCAGGGGTTC-3′; TNF-α, 5′-GGCATGGATCTCAAA GAC-3′ and 5′-AGATAGCAA ATCGGCTGACG-3′.

2.8. Other parameters

DPP-4 enzymatic activity was assayed as previously reported [17]. NO production was determined using total nitric oxide assay kit (Beyotime Institute of Biotechnology). Serum levels of glucose, triglycerides (TG) and total cholesterol (TC) were measured by automatic biochemical analyzer (Hitachi, Japan). Free fatty acid (FFA) was measured by colorimetric assays using a commercially available kit (Jiancheng, China). GLP-1, insulin, adiponectin and TNF-α in serum were measured using ELISA kits (R&D Systems, USA).

2.9. Statistical analysis

Values are expressed as mean ± SD. Statistical analysis was performed using IBM SPSS 23.0 statistics software (IBM Corp, Armonk, NY). Statistical significance was analyzed by one-way ANOVA following with Bonferroni *post hoc* tests when equal variances were assumed or Tamhane's T2 *post hoc* tests when equal variances were not assumed for comparisons between two or multiple groups. Homogeneity of variance was tested using Levene's test. *P*-value < .05

Table 1
Blood biomarkers at the end of study.

Parameter	NC	HFD	AHF
Glucose (mM)	6.1 ± 0.8	7.9 ± 2.0*	7.0 ± 1.4
Insulin (mIU)	13.25 ± 1.64	30.82 ± 2.97*	15.71 ± 2.31#
TG (mM)	1.09 ± 0.15	2.36 ± 0.17*	1.87 ± 0.23*#
TC (mM)	3.15 ± 0.11	4.82 ± 0.25*	4.78 ± 0.25*
FFA (mM)	0.53 ± 0.02	1.69 ± 0.04*	1.24 ± 0.04*#
Adiponectin (μg/ml)	11.5 ± 1.4	8.1 ± 1.5*	12.6 ± 2.2#
TNF-α (pg/ml)	32.5 ± 3.8	59.3 ± 5.2*	38.6 ± 3.9#

Data are expressed as mean ± SD. TG, triglycerides; TC, total cholesterol; FFA, free fatty acid.
* $P < .05$ *versus* NC.
# $P < .05$ *versus* HFD.

(two-sided) was considered statistically significant.

3. Results

3.1. Effects of alogliptin intervention on metabolic characteristics

Mice on high fat diet steadily gained weight during the period of study. Alogliptin intervention did not affect body weight (Supplemental Fig. 1a). As expected, DPP-4 activity trended to increase in HFD mice and alogliptin treatment significantly suppressed it (Supplemental Fig. 1b), and consequently preserved higher levels of GLP-1 in HCA mice (Supplemental Fig. 1c). Furthermore, high fat diet markedly elevated glucose, insulin, TG, FFA, TNF-α, and decreased adiponectin levels and alogliptin intervention prevented these alterations (Table 1).

3.2. Alogliptin intervention improves endothelial function

Previous study showed PVAT released several adipokines that affect vascular function in a paracrine manner [3]. In this study, we found that endothelium-dependent vasodilation in response to Ach was comparable in the presence or absence of PVAT in NC and AHF mice. However, presence of PVAT in HFD mice markedly impaired endothelium-mediated vasorelaxation response to Ach (Fig. 1a). Additionally, alogliptin intervention restored endothelial function both in the absence and presence of PVAT (Fig. 1b). Regarding the endothelium-independent vasodilation in response to SNP, no difference was observed in the presence or absence of PVAT among various groups (data not shown). Endothelium-mediated vasorelaxation is mainly mediated by increased production of NO from endothelial cells [18,19]. Thus, we measured the NO production in Krebs from aortas with PVAT in response to cumulative concentration of Ach and found HFD decreased NO production, however, alogliptin intervention attenuated this decrease (Fig. 1c).

3.3. Improvement on vasodilation function by alogliptin intervention on PVAT is GLP-1 dependent

GLP-1 triggers pleiotropic physiological effects such as activation of autophagy [12], reduction in inflammatory cytokines [11], improvement of hepatic steatosis [13]. To mimic internal environments, we added exogenous GLP-1 *ex vivo* and *in vitro*. We firstly explored whether alogliptin directly influenced PVAT and improved vascular function. To determine the optimal dosage of alogliptin and GLP-1 administration, we performed dose-response studies. Alogliptin showed no effects on vascular vasodilation at the tested dosage (Supplemental Fig. 2a). We chose low dose (50 μmol/L) in the subsequent experiments. However, GLP-1 enhanced vascular vasodilation in a concentration-dependent and saturable manner, but no differences were found between 100 nmol/L and 200 nmol/L GLP-1 (Supplemental Fig. 2b). Therefore, we selected 100 nmol/L as the optimal dosage. For *ex vivo* intervention, we found that treating PVAT from HFD mice with GLP-1 improved endothelium-dependent vasodilation function, but alogliptin alone didn't have any effect. Moreover, GLP-1 and alogliptin co-treatment showed no additive effect on improving endothelium-dependent

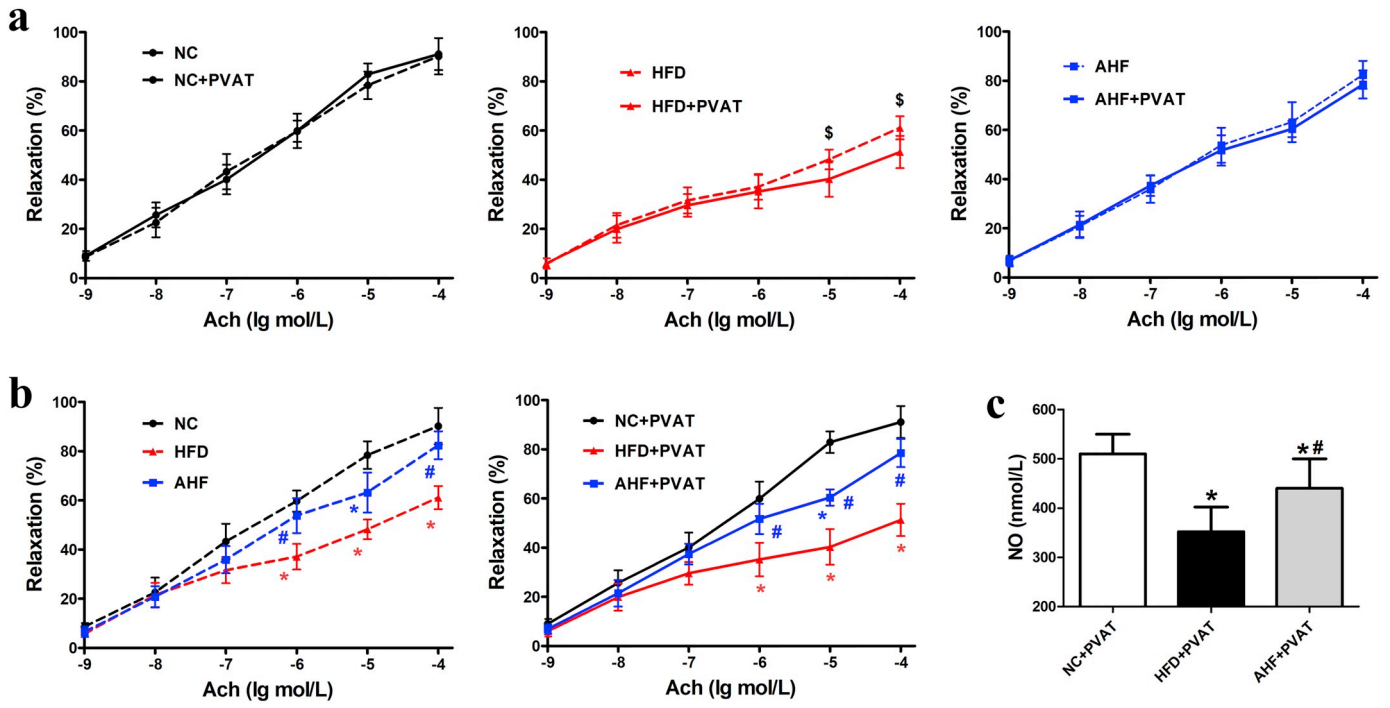

Fig. 1. Alogliptin intervention improves endothelial function. (**a**) Effects of PVAT on ACh-induced concentration-dependent vasorelaxation in various groups. (**b**) Effects of alogliptin intervention on ACh-induced concentration-dependent vasorelaxation with or without PVAT. (**c**) NO release in Krebs from aortas with PVAT in response to cumulative concentration of Ach. NC, normal chow. HFD, high-fat diet. AHF, 0.03% (wt/wt) alogliptin plus HFD. Data are expressed as mean ± SD. *$P < .05$ compared with NC group, #$P < .05$ compared with HFD group, \$ $P < .05$ compared with corresponding group without PVAT. $n = 6$ mice per group.

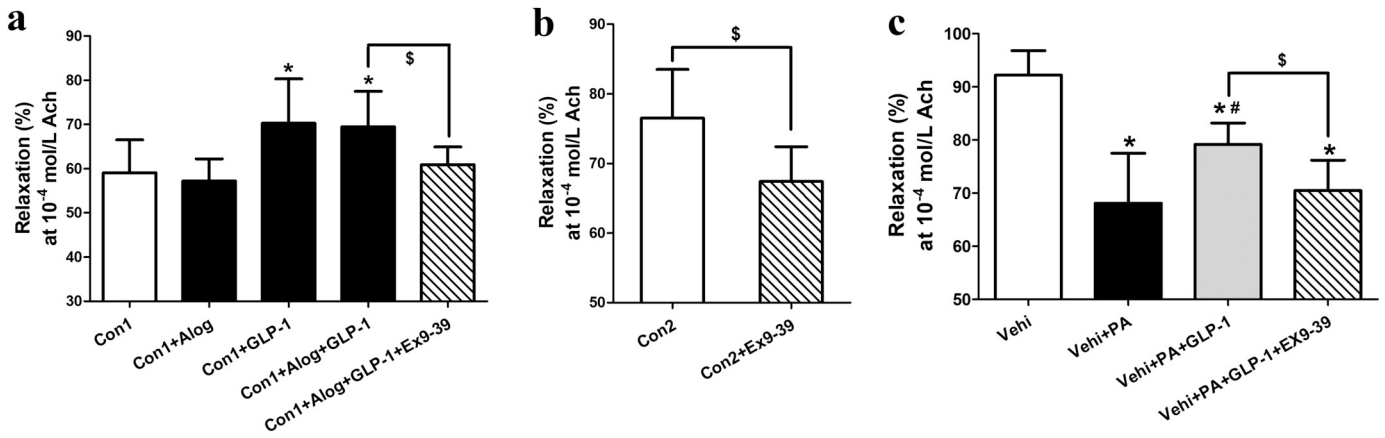

Fig. 2. Improvement in vasodilation function by alogliptin on PVAT is GLP-1 dependent. (**a**) PVAT derived from HFD mice was pretreated with Ex9-39 for 10 min, followed by addition of alogliptin for 10 min and finally GLP-1 was added for 2 h, if indicated. After washing with PBS, PVAT was incubated for 22 h. Then, the bath solution was used to incubate aorta from NC mice without PVAT for an initial 60 min and vasorelaxation function was assayed. Con 1, aorta from NC mice without PVAT incubated with bath solution of PVAT derived from HFD mice. (**b**) PVAT derived from AHF mice was pre-incubated with exendin 9-39 for 2 h. After washing with PBS, PVAT was incubated for 22 h. Then, the bath solution was used to incubate aorta from NC mice without PVAT for an initial 60 min and vasorelaxation function was assayed. Con 2, aorta from NC mice without PVAT incubated with bath solution of PVAT derived from AHF mice. (**c**) PVAT derived from NC mice was pretreated with PA for 10 min, following added Ex9–39 for 10 min and finally added GLP-1 for 2 h, where indicated. After washing with PBS, PVAT was incubated for 22 h. Then, the bath solution was used to incubate aorta from NC mice without PVAT for an initial 60 min and vasorelaxation function was assayed. NC, normal chow. HFD, high-fat diet. AHF, 0.03% (wt/wt) alogliptin plus HFD. Con, control. Alog, alogliptin. Ex9–39, exendin 9–39. Vehi, Vehicle. PA, palmitic acid. $^{*}P < .05$ compared with Con or Vehi group, $^{\#}P < .05$ compared with Vehi + PA, $\$ P < .05$. $n = 5$ mice per group.

vasodilation (Fig. 2a). We also found that treatment of PVAT derived from NC mice with PA significantly impaired endothelium-dependent vasodilation, and GLP-1 reversed this effect (Fig. 2c). In addition, these reagents didn't affect endothelium-independent vasodilation (data not shown). GLP-1 mediate its physiological effects through GLP-1R signaling [23]. Here, we found that co-treatment with GLP-1R antagonist exendin 9-39 markedly decreased endothelium-dependent vasodilation (Fig. 2a–c). Additionally, exendin 9-39 didn't affect endothelium-independent vasodilation in response to SNP (data not shown). Taken together, these results indicate that improvement on vasodilation function by alogliptin intervention on PVAT is GLP-1 dependent.

3.4. Alogliptin intervention activates autophagy in PVAT via GLP-1 dependent mechanism

Next, we explored whether alogliptin intervention activated autophagy. In adipocytes, we found MFI of red/green remarkably reduced in PA group compared to Vehicle group, indicating decreased autophagy flux. However, autophagy flux was significantly elevated by GLP-1 administration but not alogliptin alone, while autophagy flux declined sharply after exendin 9-39 exposure. Additionally, GLP-1 and alogliptin co-treatment took no additive effect on increasing autophagy (Fig. 3a–b). Together, these data indicated GLP-1 but not alogliptin alone induced autophagy.

In PVAT, analysis of microtubule-associated light chain 3B-II (LC3B-II) showed high fat diet significantly reduced LC3B-II protein level, and alogliptin intervention prevented this decrease. However, the higher LC3B-II level in AHF mice may indicate activated autophagy or inhibited autophagic maturation progress. Hence, we also measured p62 protein level to distinguish these two steps. Results showed p62 level was decreased in AHF mice compared to HFD mice, suggesting autophagy was induced in AHF mice (Fig. 3c–d). It's well known that autophagy is negatively regulated by mTOR-dependent signaling pathway [24]. We found mTOR phosphorylation increased in PVAT of HFD mice compared to NC mice and was reduced in AHF mice, indicating alogliptin intervention activated autophagy by inhibiting mTOR in PVAT (Fig. 3c–d).

3.5. Activating autophagy in PVAT is required for improving vasodilation function

To explore the effects of autophagy in PVAT on vasodilation function, we used either autophagy inducer rapamycin or autophagy inhibitor CQ to treat PVAT from HFD mice and AHF mice respectively. Results showed that PVAT bath solution from HFD mice significantly decreased endothelium-dependent vasodilation, however, rapamycin obviously attenuated this decrease. In contrast, PVAT bath solution from AHF mice markedly restored the impairment of vasodilation function compared to HFD mice, but the restoration was abolished in the presence of CQ (Fig. 3e). In accordance with *ex vivo* experiment, we also found the effects of GLP-1 on improving vasodilation function were blocked by CQ (Fig. 3f). PVAT bath solution did not affect endothelium-independent vasodilation in response to SNP in various groups (data not shown). In conclusion, these data indicate autophagy activation in PVAT is required for improving vasodilation function.

3.6. Activating autophagy in PVAT improves secreted adipokines

It has been established that PVAT undergoes dual processes of hypoxia and inflammation in obesity, leading to a reduced secretion of adiponectin and increased secretion of TNF-α which impair vascular function in a paracrine manner [3]. Thus, we further investigated the adipokines levels from PVAT after autophagy activation. There was a decrease in adiponectin level in PVAT from HFD mice compared to NC mice, while alogliptin intervention normalized adiponectin to the level of NC mice. Conversely, high fat diet elevated TNF-α level in PVAT, and alogliptin intervention prevented this effect (Fig. 4a–b). In addition, high fat diet markedly decreased adiponectin level in PVAT bath solution and this was normalized by rapamycin; while alogliptin intervention prevented the decrease in adiponectin levels and this effect was blocked by CQ (Fig. 4c). In contrast, TNF-α level was elevated in bath solution of PVAT from HFD mice and rapamycin prevented this increase; TNF-α level was not elevated in bath solution of PVAT from AHF mice but, CQ, which inhibited autophagy, trended to increase TNF-α level (Fig. 4d). Additionally, PA treatment significantly reduced adiponectin level in PVAT bath solution, while GLP-1 reversed it. However, treatment with CQ blocked the stimulatory effects of GLP-1 on adiponectin secretion (Fig. 4e). Furthermore,

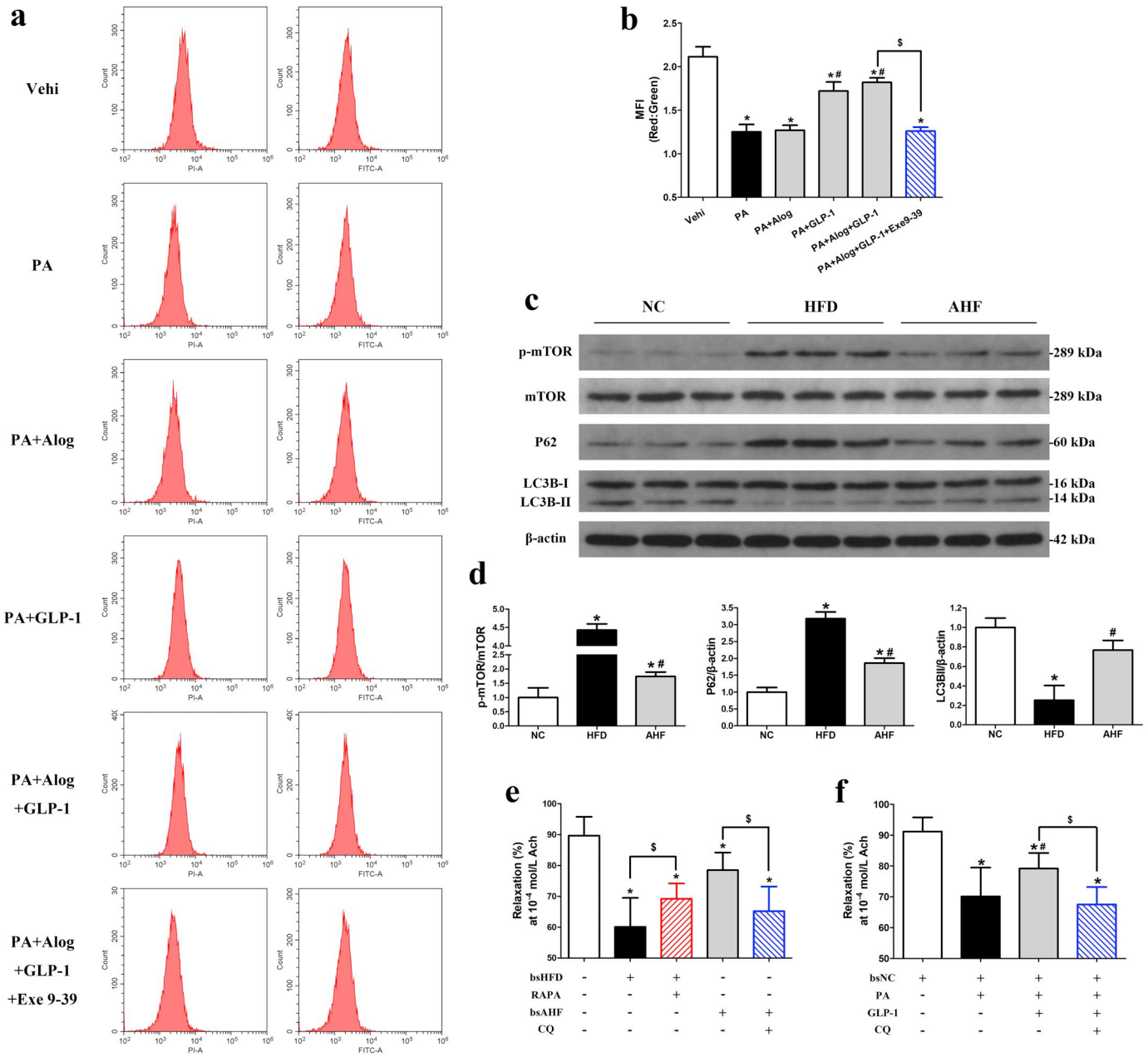

Fig. 3. Effects of autophagy in PVAT on vasodilation function. (**a–b**) Alogliptin intervention activates autophagy *in vitro*. (**a**) Adipocytes were pretreated with PA (0.4 mM) for 24 h, then added Exe 9–39 (100 nmol/L) for 30 min, following added Alog (50 μM) for 30 min, and finally added GLP-1 (100 nM), if indicated. Cells were then incubated for another 24 h and analyzed by flow cytometry. Cells were transfected with adenoviruses encoding mRFP-GFP-LC3 48 h prior to harvest. (**b**) Quantitative analysis of (**a**). (**c–d**) Alogliptin intervention activates autophagy *in vivo*. (**c**) Western blots of total adipose tissue lysates for phosphorylated (p)-mTOR, mTOR, p62 and LC3B. β-Actin was used as a loading control. (**d**) Quantitative analysis of (**c**). $n = 10$ mice per group. (**e–f**) Autophagy activation in PVAT is necessary for improving vasodilation function. **(e)** PVAT derived from HFD mice was pretreated with rapamycin (RAPA) and PVAT derived from AHF mice was pretreated with chloroquine (CQ) for 2 h as indicated. After washing with PBS, PVAT was incubated for 22 h. Then, the bath solution (bs) was used to incubate aorta from NC mice without PVAT for an initial 60 min and vasorelaxation function was assayed. (**f**) PVAT derived from NC mice was pretreated with PA for 10 min, following which CQ was added for 10 min and finally GLP-1 was added for 2 h where indicated. After washing with PBS, PVAT was incubated for 22 h. Then, the bath solution was used to incubate aorta from NC mice without PVAT for an initial 60 min and vasorelaxation function was assayed. $n = 5$ mice per group. NC, normal chow. HFD, high-fat diet. AHF, 0.03% (wt/wt) alogliptin plus HFD. PA, palmitic acid. Alog, alogliptin. Exe 9–39, exendin 9–39. MFI, mean fluorescence intensity. Data are expressed as mean ± SD.*$P < .05$ compared with Vehi or NC group, $^{\#}P < .05$ compared with PA or HFD group, $ $P < .05$.

TNF-α level in PVAT bath solution was significantly elevated after PA exposure and GLP-1 prevented this increase. However, the inhibitory effect of GLP-1 on TNF-α was blocked by CQ (Fig. 4f). We also confirmed the effects of autophagy on adipokine secretion in adipocytes and observed a decrease in adiponectin expression and an increase in TNF-α expression in PA treatment, and GLP-1 reversed these effects. However, in the presence of CQ, the effects of GLP-1 were attenuated (Fig. 4g–h). Taken together, our results indicate that activating autophagy in PVAT increased adiponectin level and decreased TNF-α. The altered adipokines secretion, at least partly, mediated improvement in vascular function.

3.7. PVAT bath solution affects NO production via Akt-eNOS signaling pathway in vitro

Endothelium-mediated vasorelaxation is mainly mediated *via* PI3K-Akt pathway resulting in phosphorylation of eNOS and consequently

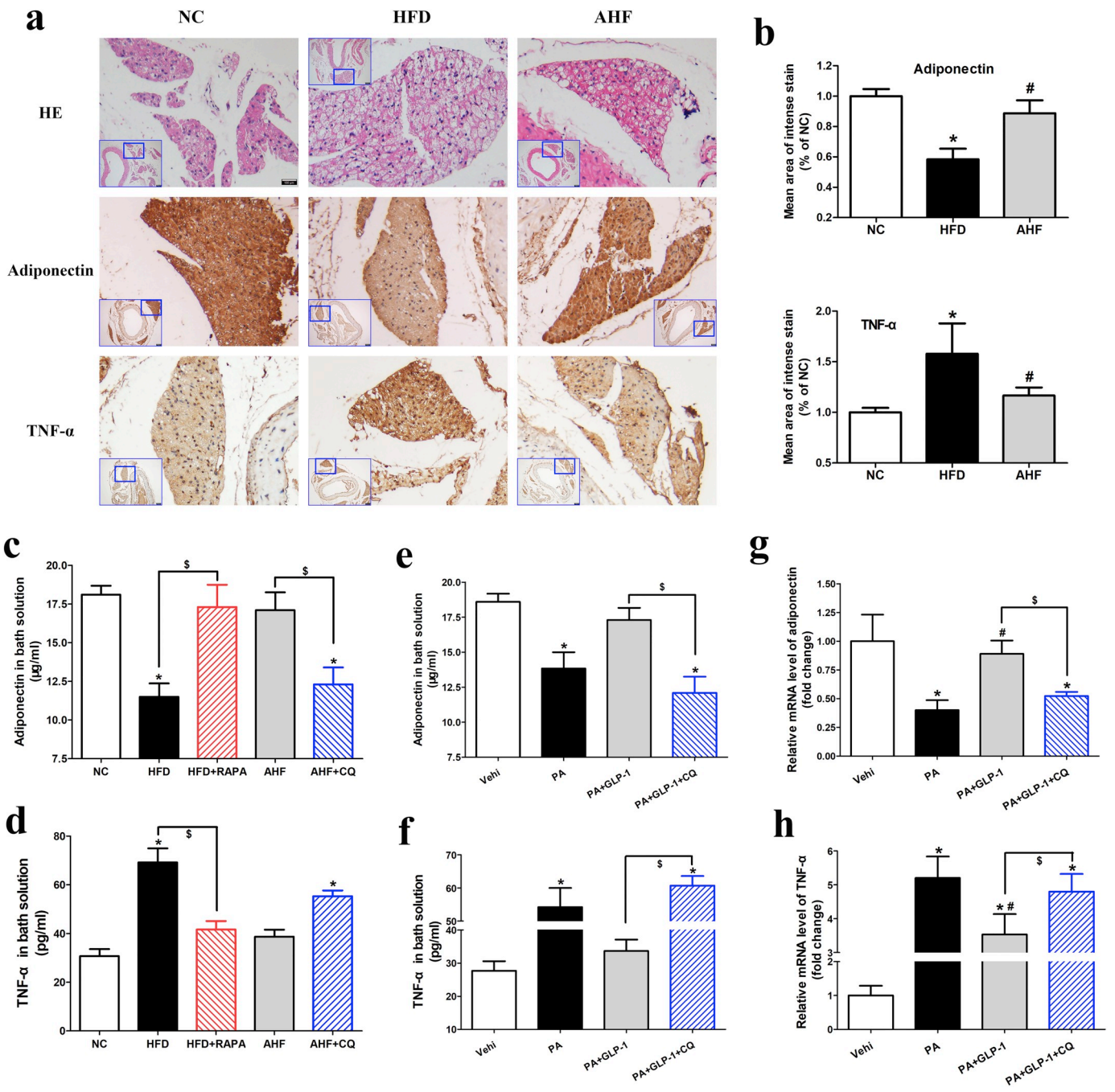

Fig. 4. Effects of autophagy in PVAT on adipokines expression. (**a**) HE staining, immunohistochemical detection for adiponectin and TNF-α in various groups. Scale bar, 100 μm. $n = 5$ mice per group. (**b**) Quantitative analysis of adiponectin and TNF-α. (**c-f**) Concentration of adiponectin and TNF-α in bath solution at the presence of autophagy activator or inhibitor. (**g-h**) Adipocytes were pretreated with PA (0.4 mM) for 24 h, then CQ (20 μmol/L) was added for 30 min, followed by GLP-1 (100 nM) where indicated. Cells were further incubated for another 24 h. Relative mRNA level of adiponectin (**g**) and TNF-α (**h**) were determined by qPCR. NC, normal chow. HFD, high-fat diet. AHF, 0.03% (wt/wt) alogliptin plus HFD. Vehi, Vehicle. PA, palmitic acid. CQ, chloroquine. RAPA, rapamycin. Data are expressed as mean ± SD. $^{*}P < .05$ compared with NC or Vehi group, $^{\#}P < .05$ compared with HFD or PA group, $\$ P < .05$.

increased NO production [25]. We further characterized the possible mechanism of endothelium protective effect mediated by PVAT secretion. Bath solution of PVAT derived from HFD mice inhibited phosphorylation of Akt and eNOS as well as significantly decreased NO production in human umbilical vein endothelial cells (HUVECs), while PVAT bath solution derived from AHF mice obviously attenuated these effects. Moreover, the activated Akt was blocked by LY294002, and the activated eNOS as well as increased NO production were blocked in the presence of LY294002 and L-NAME (Fig. 5a–d). Collectively, these data indicated PVAT secretion affects endothelium function, at least partly, through activating Akt- eNOS signaling pathway.

4. Discussion

Our data for the first time suggested that alogliptin intervention activates autophagy in PVAT and improves endothelial function in mouse model of obesity. The major findings include (1) alogliptin intervention improved vasodilation function by enhancing autophagy

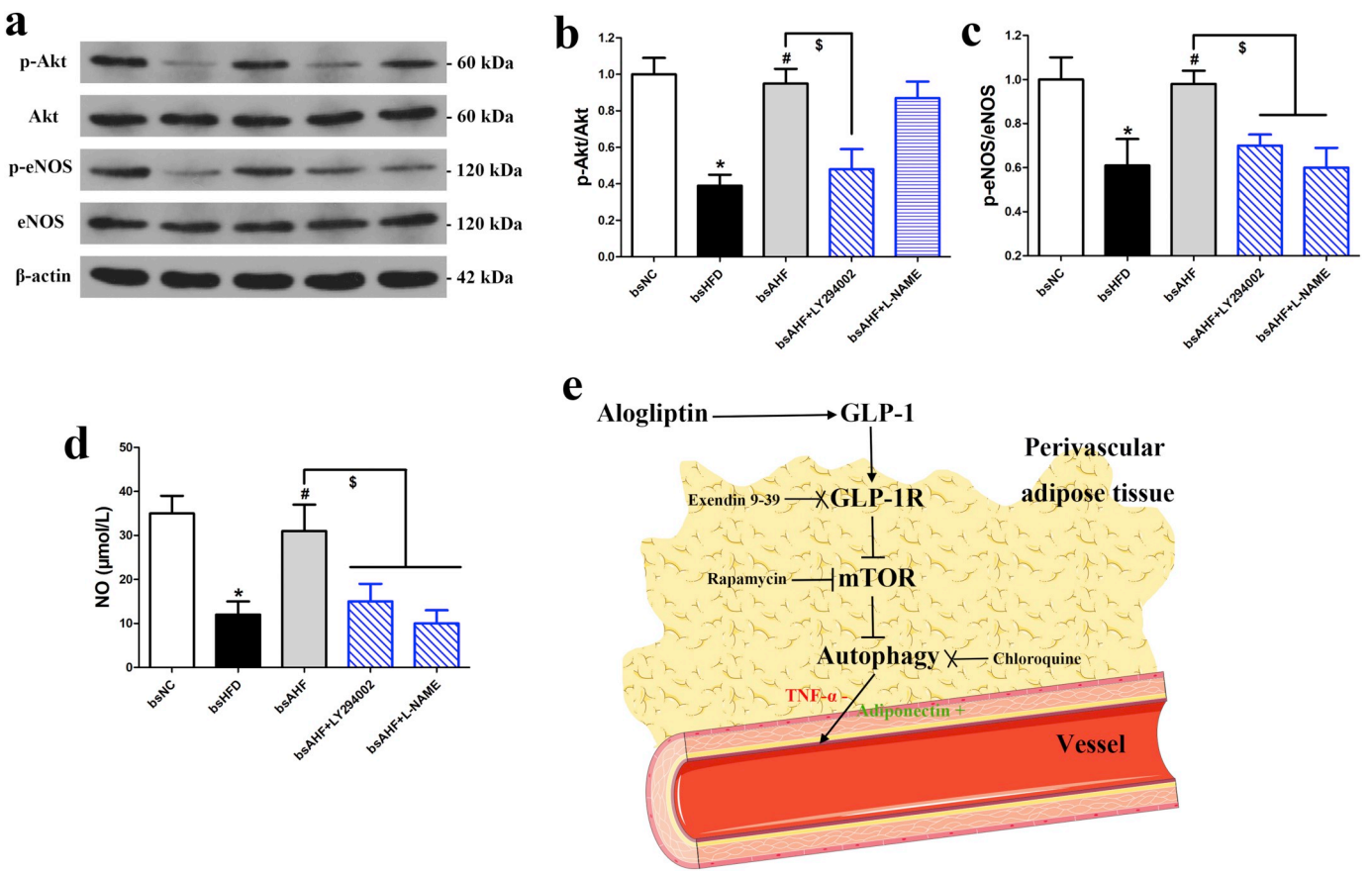

Fig. 5. PVAT affects Akt-eNOS signaling pathway and NO production *in vitro*. (**a-d**) HUVECs were pretreated with PI3K inhibitor LY294002 (5 μmol/L) or eNOS inhibitor L-NAME (100 μmol/L) 30 min before PVAT bath solution (bs) treatment, after incubation for another 2 h, cells were harvested for Western blotting and culture medium was collected for measuring NO production. Western blots (**a**) and quantitative analysis for phosphorylated (p)-Akt (**b**) and p-eNOS (**c**). (**d**) NO production in culture medium. (**e**) Schematic diagram of alogliptin intervention. NC, normal chow. HFD, high-fat diet. AHF, 0.03% (wt/wt) alogliptin plus HFD. Data are expressed as mean ± SD. $^{*}P < .05$ compared with NC group, $^{\#}P < .05$ compared with HFD group, $^{\$}P < .05$.

level in PVAT of obese mice; (2) the beneficial effects of alogliptin intervention on PVAT were GLP-1 dependent (Fig. 5e).

Obesity, due to an imbalance between energy intake and expenditure, has become a global public heath challenge. Obesity is also a risk factor for vascular disfunction and cardiovascular disease [26,27,28,29,30]. In the present study, we found high fat diet decreased endothelium-dependent vasodilation and alogliptin intervention attenuated this effect. However, the underlying mechanisms of alogliptin intervention were less clear. Previous study [31] showed that sitagliptin, another DPP-4 inhibitor, improved endothelial function and reduced atherosclerotic lesion formation by augmenting GLP-1 activity in endothelium. Here, we focused on the effects of PVAT on vascular function. PVAT, a special type of adipose tissue, secrets cytokines to regulating vascular tone. Recent studies have demonstrated that PVAT is an important source of oxidative stress and inflammation in obesity which may result in vascular disfunction [29,32,33]. In our study, PVAT significantly reduced endothelium-mediated vasodilation in respond to Ach in HFD mice, but no discernible difference in endothelium-dependent vasodilation was observed with or without PVAT in alogliptin intervention. Moreover, alogliptin intervention increased adiponectin level and decreased TNF-α level from PVAT, which at least in part, contributed to improvement in vascular vasodilation. These results are congruent with previous work [34] showing that DPP-4 inhibitor teneligliptin inhibited atherogenesis in normoglycemic apolipoprotein-E-deficient mice by attenuating pro-inflammatory phenotype of PVAT.

Autophagy is a critical intracellular catabolic pathway which degrades various substrates to support energy balance. In addition, basal level of autophagy is necessary for maintaining the biological functions of organs. Previous studies have shown that GLP-1 related interventions improved functions of several organs by activating autophagy [12,14,35,36,37,38,39]. Here, we considered the enhanced autophagy level in PVAT as an underlying mechanism for the improving endothelial function by alogliptin intervention based on the following results: (1) The autophagy level in PVAT was reduced on high fat diet, and alogliptin intervention attenuated this decrease *in vivo*; (2) Elevating autophagy level in PVAT of HFD mice using rapamycin improved endothelium-dependent vasodilation *ex vivo*. In contrast, blocking autophagy in PVAT of AHF mice using chloroquine impaired the restored endothelium-mediated vasodilation by alogliptin intervention *ex vivo*; (3) *In vitro*, GLP-1 attenuated PA-induced endothelium-dependent vascular disfunction, which was suppressed by chloroquine; (4) Activating autophagy increased adiponectin level and reduced TNF-α level in PVAT both *in vivo* and *ex vivo*. Autophagy is dominantly activated by mTOR-related signaling [40]. In present study, we found mTOR was activated, and autophagy was inhibited on high fat diet, while alogliptin intervention inhibited mTOR and consequently activated autophagy.

DPP-4 inhibitors are commonly prescribed drugs for treatment of patients with type 2 diabetes. They exert glucose-dependent hypoglycemic effect mainly by increasing active GLP-1 levels. However, previous study [41] found that pharmacological inhibition of DPP-4 enhanced endothelial growth independent of GLP-1. Here, we determined whether alogliptin directly influenced PVAT independent of elevating GLP-1 level and found alogliptin administration alone showed no effects. It is believed that GLP-1R mediates the broad pleiotropic effects of

GLP-1 [12,42,43]. However, some study has described that cardiac and vascular actions of GLP-1 were mediated through both GLP-1R dependent and independent mechanisms [44]. In the present study, we wondered whether the beneficial effects of GLP-1 in PVAT were regulated *via* GLP-1R signaling. Results showed that the improved vasodilation function was abolished in exendin 9-39 administration, a GLP-1R antagonist, indicating GLP-1R was necessary for downstream effects of GLP-1.

Our study has some limitations. Firstly, in our study, autophagy reactivation in PVAT is essential to improve PVAT function, but we do not further explore how does autophagy restoration affect transcription and secretion of cytokines in PVAT. Some studies found autophagy inhibition led to inflammatory responses and insulin resistant *via* endoplasmic reticulum (ER) stress [45,46]. Further studies are required to explore whether alogliptin intervention decreases ER stress and thereby attenuates inflammation in PVAT. Secondly, alogliptin was reported to impair endothelial function in type 2 diabetes [47]. Here, we did not perform relevant human study. So, more evidences are needed to generalize the beneficial effects of alogliptin intervention on PVAT from mouse to human.

5. Conclusions

Collectively, the present study opens new mechanistic insights into the improvement of endothelial dysfunction in obesity by alogliptin intervention. GLP-1, which is elevated by alogliptin intervention, activates autophagy in PVAT, and consequently alters adipokines secretion, thereby contributes to improvement in vascular function.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vph.2018.11.003.

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Author disclosure statement

No competing financial interests exist.

Author's contributions

B.Z., W.M., M.H. and Y.D. conducted the animal experiments. Y.D., W.M., B.M. and H.Z. performed the *in vitro* experiments. B.Z., Y.L., and G.X. analyzed the data and wrote the manuscript. G.X. is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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